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13. ABSTRACT *(Maximum 200 words)*

PURPOSE: The purpose of this study is to use sentinel node technology to better predict prognosis for node negative patients.

SCOPE: Patients with a new diagnosis of unilateral, unifocal breast cancer and a clinically negative axilla were offered enrollment in this protocol. Sentinel nodes were localized and resected along with the standard staging axillary dissection. The sentinel nodes were processed both in the standard fashion and were saved for specialized studies including microsectioning and telomerase studies to be carried out in a delayed fashion by protocol requirement. Short-term goals are (1) to determine the proportion of patients considered node negative who harbor micrometatasis or telomerase in the sentinel node, and (2) to determine the association of nuclear grade and Ki-67 with the finding of micrometastasis in the sentinel node. Long-term goals include evaluation of the association of sentinel node micrometastasis and telomerase with disease free survival and overall survival.

MAJOR FINDINGS: In the time interval covered by this report, a total of 86 patients entered this study ranging in age from 38 to 87. All enrolled patients have been female. Both short-term outcomes and long-term outcomes are pending at this time as required by the protocol.

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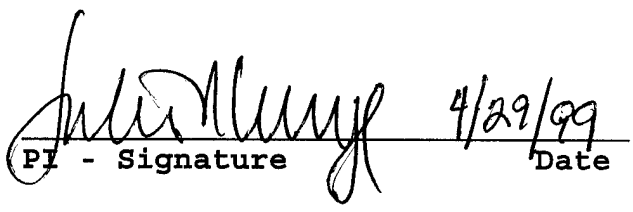

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V. INTRODUCTION

Nature of Problem: Today with increased public awareness of breast cancer and increased usage of screening mammography, approximately 60% of newly diagnosed breast cancer patients are found to be node negative. (1) However, node negative patients represent a group with a heterogeneous prognosis. While the majority of these patients will be cured by local therapy alone, approximately 25-30% will go on to recur and die of their disease within 10 years of the time of the diagnosis. (2,3,4) A smaller proportion will succumb to their disease more than ten years after diagnosis. At present our ability to predict which of the node negative group of patients will have a poor outcome is quite limited. Features of the primary tumor such as size, differentiation, estrogen receptor (ER) and progesterone receptor (PR) status while useful are of limited predictive value. It is difficult to identify which patients are likely to benefit from adjuvant therapy and those who may safely forego adjuvant therapy. Many patients who are currently node negative by standard processing are offered adjuvant therapy on the basis of the characteristics of the primary tumor. Node negative patients are felt to accrue a benefit of approximately 10% in absolute risk reduction with standard adjuvant chemotherapy or tamoxifen therapy. (5,6) However, approximately 70% of node negative patients can be expected to do well without adjuvant therapy. (7) It is likely that a significant proportion of node negative patients who would not have recurred without adjuvant now receive adjuvant therapy from which they cannot benefit. These patients however do incur the morbidity and the financial cost of adjuvant therapy. It is currently very difficult to predict which node negative patients are likely to recur and therefore stand to benefit from adjuvant therapy and which patients can safely forgo adjuvant therapy.

Background of Previous Work: Sentinel lymph node biopsy is an emerging technique for the evaluation of regional lymph nodes in patients with newly diagnosed cancers. It is based on the idea that lymphatic drainage from a given primary tumor site will go first preferentially to a specific and identifiable lymph node (the sentinel) and that if that node is negative the remainder of the nodes are likely to be negative as well. It is under widespread and intense investigation for use in breast cancer (8,9). The technique may within the next few years become the standard of care for patients with newly diagnosed breast cancer. One advantage of the sentinel node biopsy is that it could provide

staging with less morbidity than a standard staging axillary dissection. Another advantage is that it provides one or two lymph nodes which can then be subjected to more extensive pathologic review than is typically done with nodes from a standard staging dissection. Therefore sentinel node biopsy may be a technique which can help provide a more accurate microstaging of the regional node basin and may therefore provide additional information not provided by a standard staging axillary dissection.

For patients with newly diagnosed breast cancer the status of the regional nodes is the single most important prognostic factor. For those patients considered to be node negative, the possibility of unidentified micrometastasis within lymph nodes may be one feature which would predict with a poorer outcome. Lymph node micrometastases are defined as nodal metastasis identifiable on hematoxylin and eosin (H&E) staining that are less than 2 mm in size. Micrometastases are known to be missed on routine histologic sectioning in approximately 10-30% of cases. (10,11) The significance of micrometastases has been debated. One large study reported the presence of micrometastases made no difference in outcome. (12) However, other studies have found a somewhat poorer prognosis in these "node negative" patients who are found to have nodal micrometastasis. (13,14,15)

In recent years there has been a great deal of interest in the potential usefulness of telomerase as a means of identifying malignant cells. Telomerase is a ribonucleoprotein which is telomere specific and adds nucleotide repeats to chromosome ends. Telomerase is known to be active only in cells with proliferative capacity such as germ line cells, stem cells and malignancies (16,17,18). It is not active in normal somatic cells. Telomerase is under active investigation as a way of identifying malignant cells in breast cancer. It is now possible with a polymerase chain reaction (PCR)-based assay to detect evidence of telomerase in samples as small as 10-100 cells. (19,20)

Methods of Approach: This protocol uses sentinel node technology to identify and selectively remove the sentinel node as a separate specimen from the remainder of the axillary dissection. The sentinel node is saved in a planned fashion for delayed processing for serial sectioning for micrometastases and telomerase studies. The pathologic processing of the sentinel node specimen calls for immediate processing as a standard lymph node for pathologic review including one or two sections per block with

routine H&E stains. The blocks are saved and at delayed interval are serially sectioned at 8 micron intervals. They are examined using H&E staining. A tiny portion of the sentinel node is saved for telomerase studies using a PCR-based telomeric repeat amplification protocol (TRAP). (19,20) These are also processed in a delayed fashion. Short-term outcomes include association of characteristics of the primary tumor with the finding of micrometastasis and telomerase in the sentinel node. The long-term outcomes of this study are the association between micrometastasis or telomerase in the sentinel node and disease free and overall survival.

VI. BODY

Experimental Methods

Protocol Design. The design is a prospective cohort study. Patients with newly diagnosed early stage breast cancer were offered enrollment and underwent a sentinel lymph node biopsy as part of their surgical staging as well as a standard level I and II axillary dissection. The sentinel lymph nodes were evaluated both with routine histopathology as currently practiced and also with specialized techniques to look for evidence of microscopic disease that may not be appreciated on routine sectioning. The decision to offer a patient adjuvant therapy was based on the pathologic findings of the primary tumor and the standard evaluation of the lymph nodes. The findings from the specialized studies were not introduced into the clinical chart. Patients are then to be followed to determine their disease free survival and overall survival.

Patient Population. The Johns Hopkins Breast Center and the Johns Hopkins Greenspring Station Breast Center are the sites for recruitment of patients. Inclusion criteria include 1) Newly diagnosed operable invasive breast cancer. 2) Clinically negative ipsilateral axilla. 3) Patients for whom a staging axillary lymphadenectomy is considered the current standard of care. 4) Primary tumor size estimated by clinical and radiographic criteria to be less than or equal to three centimeters. 5) Willing participation following informed consent. Exclusion criteria are: 1) Clinically positive lymph nodes. 2) Pregnancy. 3) Previous axillary lymphadenectomy, 4) Multifocal or bilateral disease. Patients were registered at the time of their informed consent and were assigned consecutive identification numbers.

Statistical Methods. The sample size needed was calculated based on the survival endpoint, and is estimated to be approximately two hundred and sixty patients assuming an alpha error of 0.05, beta error of 0.2. This assumes that seventy percent of patients enrolled will be node negative, that twenty five percent of those who are node negative will be micrometastasis positive and that there will be a twenty percent difference in disease free survival at five years between the groups that are micrometastasis positive and micrometastasis negative. This sample size provides eighty percent power to determine the difference between seventy percent and ninety percent disease-free survival at five years.

Study Procedure. Technetium – 99M Sulfur colloid is prepared by the radiopharmacy at Johns Hopkins for each case. The material is used unfiltered. A total of 0.95 mCi of

Technetium is diluted into a final volume of 4.0 ml. A total of 4.0 ml of the radiotracer is injected into the breast of each patient enrolled; 1.0 ml per injection site is injected into normal tissue superior, inferior, medial and lateral to the primary cancer or biopsy cavity. At each injection site the 1.0 ml volume is injected from the deepest to the most superficial level of the tumor or biopsy cavity within the breast. The injection is guided by ultrasound or stereotactic mammography whenever possible. The injection is performed by personnel certified in the handling of radioactive substances. Precautions are maintained according to radiation safety guidelines of handling open containers of radiopharmaceutical. All materials used for injection (syringes, gloves, gauze used to wipe the skin or spills) are handled and disposed by the personnel of the nuclear medicine department. A total of 0.95 mCi of technetium is the total amount injected per patient. The time interval between injection and surgery is from one to six hours. The gamma detector used intraoperatively is the C-Trak gamma detector (Carewise Medical, Morgan Hill, CA.) . The settings used are a threshold of 130 keV and a window of 40 keV. All counts are accumulated over 10 seconds intraoperatively.

The sentinel lymph node biopsy is performed at the same time the patient is scheduled for a staging axillary lymphadenectomy. The patient is brought to the operating room approximately one to six hours following the injection of the radioisotope. The procedure is performed as it would be in the absence of a sentinel node biopsy. In the course of the procedure when the axilla is first entered either during the course of a modified radical mastectomy or an axillary dissection, the hand held gamma probe is used intraoperatively to localize the sentinel lymph node. This lymph node is dissected from surrounding tissues and removed as a separate specimen.

In the operating room the sentinel lymph node is dissected by the surgeon, two touch preps or tiny silvers of tissue are prepared on glass slides and the glass slides dropped into liquid nitrogen for telomerase studies. The sentinel lymph node is then labeled and sent to surgical pathology.

The remainder of the patient's primary surgical therapy for breast cancer is completed at the same setting, including a standard level one and level two axillary lymph node dissection.

Telomerase. The slides of the touch preps made in the operating room from the sentinel lymph node are briefly thawed and overlaid with thirty-five microliters of CHAPS lysis buffer. Cells are scraped off with the pipette tip and the lysate collected. The five microlite aliquot is used for assay of total protein and the remainder of the sample is

allocated and snap frozen in liquid nitrogen. The PCR-based telomeric repeat amplification protocol (TRAP) is performed as described (19,20) with the following exceptions: 10 ag of a PCR control (ITAS) is added to the reaction mixture, the reactions will be incubated for 45 minutes, and the annealing temperature used is 56°. Each cell extract is accompanied by the same cell extract 1) inactivated by heating to 94°C for ten minutes and assayed as a negative control and 2) assayed in a concurrent PCR using primer for a housekeeping gene, RPA, as a positive control for a cellular aspirate, since the amount of protein in the lysate is often too low to be assayed by standard techniques. The RPA primers amplify under the same reaction conditions as the telomerase primers. The TRAP assay is scored in a binary fashion with a positive result defined as any banding pattern (laddering) beyond background.

Processing of the Sentinel Node in Surgical Pathology. Upon receipt by surgical pathology the sentinel node(s) is processed and embedded in a routine fashion after appropriate gross inspection of the specimen. One section will be made per block. Routine hematoxylin stains is done and the remainder of the paraffin block will be saved for future review. At an interval of four to six months recently accumulated sentinel node blocks are processed in the surgical pathology research laboratory in a batch fashion. The blocks are serially sectioned at 8 micron intervals. All paraffin ribbons are collected, mounted on slides and examined for evidence of micrometastasis using standard H&E stain.

Surgical Pathology of the Primary Breast Cancer and Axillary Contents. The breast specimen whether it be mastectomy or partial mastectomy is processed in the routine fashion for definition of the exact pathologic size of the tumor and histologic evaluation. The primary tumor is evaluated in the standard fashion and analyzed for histologic grade according to the Elston grading system. The tumor is analyzed for Ki-67, as is currently routine. The axillary contents are processed in a routine fashion with dissection of individual lymph nodes and processing of each node with one or two sections with H&E staining.

Follow Up. Patients are followed for a minimum of five years and for longer whenever possible to determine disease free survival and overall survival. Disease free survival is defined as ending at the first diagnosis of distant (not locoregional) disease. Patients enrolled in this protocol are screened according to the Johns Hopkins breast follow up protocols which include at least every six-month evaluation by a physician.

Pathologic Outcomes. Sentinel lymph nodes that are negative for metastases by routine histology are assessed for micrometastases and telomerase. The proportion of these nodes found to be positive for micrometastases or telomerase is calculated. The nuclear grade (Elston grading) or a primary tumor and Ki-67 in the primary tumor is tested for association with the finding of micrometastasis in the sentinel lymph as an indirect measure of outcome in two by two tables using the Chi square test.

Survival Outcomes. Patients who prove to be node negative by routine histologic criteria will be followed for a minimum of five years to determine disease free survival. Disease free survival is then analyzed according to the presence or absence of micrometastases and according to the presence or absence of telomerase in the sentinel lymph node. Factors controlled for all the patients menopausal status, the size of the primary tumor and whether or not the patient received adjuvant therapy. Statistical modeling are done according to the Cox proportional hazards model to estimate the risk ration attribute to micrometastases and telomerase on disease-free survival and overall survival.

Results

During the time interval covered by this report, 86 patients were entered in this protocol. All are female. They range in age from 38 to 87. All patients had a unilateral, unifocal lesion estimated to be less than 3 cm in greatest dimension and had a clinically negative axilla. Each gave voluntary, informed consent. Protocol procedures were followed for each patient. Each patient underwent a peritumoral injection of approximately 0.95 mCi of Technetium sulfur colloid. In the operating room the gamma probe was used to localize and assist with the resection of the sentinel node as a separate specimen and the specimens were processed as outlined above.

One complication directly related to participation in this protocol was encountered. This was reported in detail to our Institutional Review Board and also reported to the Department of Defense in March, 1998. Briefly, an enrolled patient had a minimal pneumothorax as a result of the placement of the injection needles. This was discovered intraoperatively. The patient was observed overnight. She was asymptomatic and the pneumothrax re-absorbed spontaneously. The patient was seen in March of 1999 by her surgeon in routine follow-up and has had no long-term sequelae.

The pathologic endpoints are underway and are not ready for reporting at this time. By design of the protocol the study outcomes are processed in a delayed fashion. My expectation is that they will be ready for reporting in another six months. Long-term outcomes of disease free survival and overall survival will slowly be accrued over the next several years and should be available for report in approximately five years.

Discussion

The protocol is continuing according to the outline of Statement of Work in the original proposal.

Task 1 has been completed. This includes an initial enrollment of patients. The principal investigator was present for and monitored the sentinel node biopsy and handling of the sentinel node for the first ten cases of each participating surgeon. Frequent discussion with collaborators from Dr. Sukumar's lab and Nuclear Medicine and Pathology was adhered to.

Task 2 is ongoing. Patients are continuing to be enrolled according to the outlined protocol.

Task 3 Dr. Sukumar's lab has been performing the telomerase studies without difficulty. Data is maintained separate from the clinical record. These studies are still underway and will be ready for reporting in another six months or so.

Task 4 Microsectioning of the sentinel lymph node in surgical pathology takes place in a batch fashion every 4-6 months. Information obtained from specialized processing is maintained separately from the clinical record. I expect that this information will be ready for an initial report in approximately 6 months.

Task 5,6 and 7 are ongoing with further patient accrual and analysis of the pathologic endpoints.

Task 8 including follow-up for survival outcome by necessity must be completed over the next several years.

VII. CONCLUSION

This study is ongoing and conclusions cannot be drawn at this time. I expect that short-term pathologic endpoints will be available for discussion in approximately six months and longer-term survival endpoints will be available in approximately five years.

VIII. REFERENCES

1. Ries LAG, Miller BA, Hankey BF, et.al.,eds. SEER cancer statistics review, 1973-1991: tables and graphs. National Institutes of Health publ no. 94-2789. Bethesda, MD, USDHHS National Cancer Institute, 1994.

2. Haagensen C. Treatment of curable carcinoma of the breast. Int J Radiat Oncol Phys 1977;2:975.

3. Valagussa P, Bonadonna G, Veronesi V. Patterns of relapse and survival following radical mastectomy. Cancer 1978; 41:1170.

4. Ferguson D, Meier P, Karrison T, et.al. Staging of breast cancer and survival rates: an assessment based on 50 years of experience with radical mastectomy. JAMA 1982;248:1337.

5. Fisher B, Redmond C, Dimitrov NV, et.al A randomized clinical trial evaluating sequential methotrexate and fluorouracil in the treatment of patients with node-negative breast cancer who have estrogen-receptor-negative tumors. N Engl J Med 1989;320:473.

6. Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. Lancet 1992;339:1.

7. Rosen PP, Groshen S, Saigo PE, et.al. A long-term follow-up study of survival in stage I ($T_1N_0M_0$) and stage II ($T_1N_1M_0$) breast carcinoma. J Clin Oncol 1989;7:355.

8. Guiliano AE, Kirgan DM, Guenther JM, et.al. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. Annals of Surgery 1994;220:391.

9. Krag DN, Weaver DL, Alex JC, et.al. Surgical resection and radiolocalization of the sentinel lymph node in breast cancer using a gamma probe. Surgial Oncology 1993;2:335.

10. Saphir O, Amronin G. Obscure axillary metastasis in carcinoma of the breast. Cancer 1948;1:238.

11. Fisher E, Swamidoss S, Lee C, et.al. Detection and significance of occult axillary node metastases in patients with invasive breast cancer. Cancer 1978;42:2025.

12. Rosen P, Saigo P, Braun D, et.al. Axillary micro- and macro-metastases in breast cancer: prognostic significance of tumor size. Ann Surg 1981;194:585.

13. deMascarel I, Bonichon F, Coindre J, et.al. Prognostic significance of breast cancer axillary lymph node metastases assessed by two special techniques: reevaluation with longer follow-up. Br J Cancer 1992;66:523.

14. International (Ludwig) Breast Cancer Study Group. Prognostic significance of occult axillary lymph node micrometastases from breast cancers. Lancet 1990;335:1565.

15. Friedman S, Bertin F, Mouriesse H, et.al. Importance of tumor cells in axillary node sinus margins ("clandestine" metastases) discovered by serial sectioning in operable breast cancer. Acta Oncol 1988;27:483.

16. Greider CW. Telomerase and telomere-length regulation: lessons for small eukaryotes to mammals (Review) Cold Spring Harbor Symposia on Quantitative Biology 1993;58:719.

17. Kim NW, et.al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011.

18. Counter CM, Avilion AA, LeFeuvre CE, et.al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 1992;11:1921.

19. Piatyszek MA et.al. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). Methods Cell Sci 1995;17:1.

20. Wright WE, et.al. Modification of a telomeric repeat amplification protocol (TRAP) results in increased reliability, linearity, and sensitvity. Nucleic Acids Res 1995;23:3794.